DEPARTMENT OF THE AIR FORCE
59TH MEDICAL WING (AETC)
JOINT BASE SAN ANTONIO - LACKLAND TEXAS

5 DEC 2016

MEMORANDUM FOR ST
ATTN: HUI XIA

FROM: 59 MDW/SGVU

SUBJECT: Professional Presentation Approval

1. Your paper, entitled **A Multiplex Quantitative Analysis of Secreted Proteins in Bronchoalveolar Lavage Samples from War Veterans with Chronic Respiratory Symptoms** presented at/published to **Poster: A Multiplex Quantitative Analysis of Secreted Proteins in Bronchoalveolar Lavage Samples from War Veterans with Chronic Respiratory Symptoms** in accordance with MDWI 41-108, has been approved and assigned local file #**16395.**

2. Pertinent biographic information (name of author(s), title, etc.) has been entered into our computer file. Please advise us (by phone or mail) that your presentation was given. At that time, we will need the date (month, day and year) along with the location of your presentation. It is important to update this information so that we can provide quality support for you, your department, and the Medical Center commander. This information is used to document the scholarly activities of our professional staff and students, which is an essential component of Wilford Hall Ambulatory Surgical Center (WHASC) internship and residency programs.

3. Please know that if you are a Graduate Health Sciences Education student and your department has told you they cannot fund your publication, the 59th Clinical Research Division may pay for your basic journal publishing charges (to include costs for tables and black and white photos). We cannot pay for reprints. If you are 59 MDW staff member, we can forward your request for funds to the designated wing POC.

4. Congratulations, and thank you for your efforts and time. Your contributions are vital to the medical mission. We look forward to assisting you in your future publication/presentation efforts.

PAUL T. BARNICOTT, GS-15-DAF
Deputy Director, Clinical Research Division

PROCESSING OF PROFESSIONAL MEDICAL RESEARCH PUBLICATIONS/PRESENTATIONS

INSTRUCTIONS

1. The author must complete page two of the 59 MDW Form 3039 (this form):

   a) In Section 2, add the funding source for your study [e.g., 59 MDW CRD Graduate Health Sciences Education (GHSC) [SG5 O&M]; SG5 R&D; Tri-Service Nursing Research Program (TSNRP); Defense Medical Research & Development Program (DMRDP); NIH; Congressionally Directed Medical Research Program (CDMRP); Grants; etc.]

2. Print your name, rank/grade, sign and date the form in the author's signature block or use electronic signature.

3. Attach a copy of the 59th MDW IRB or IACUC approval letter for the research related study. If this is a technical publication/ presentation, state the type (e.g., case report, QA/QI study, program evaluation study, informational report/briefing, etc.) in the "Protocol Title" box of the 59 MDW Form 3039.

4. Attach a copy of your abstract, paper, poster and other supporting documentation.

5. Save and forward, via email, the processing form and all supporting documentation to your Unit Commander, Program Director or immediate supervisor for review/approval.

6. On page 2, have either your Unit Commander, Program Director or immediate supervisor:

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7. Contact the 59th CRD/Publications and Presentations Section at (292-7141) for instructions for submitting the request form.

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NOTE: All abstracts, papers, posters, etc., should contain the following disclaimer statement:

"The views expressed are those of the [author(s)] [presenter(s)] and do not reflect the official views or policy of the Department of Defense or its Components."

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"The experiments reported herein were conducted according to the principles set forth in the National Institute of Health Publication No. 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended."

PROCESSING OF PROFESSIONAL MEDICAL RESEARCH PUBLICATIONS/PRESENTATIONS

TO:	FROM: Author's Name, Rank, Grade, Office Symbol	PROTOCOL NUMBER:
Clinical Research Division/SGVU (59 MDW/SGVU)	Hui Xia, CTR, 59MDW/ST	C.2011.203 / 369847

PROTOCOL TITLE - ***[NOTE: For each new release of medical research or technical information as a publication/presentation, a new 59 MDW Form 3039 must be submitted for review and approval.]***
Study of Active Military Personnel for Pulmonary Disease Related to Environmental Dust Exposure – 13 Comprehensive Dyspnea Evaluation (STAMPEDE-CDE)

1. TITLE OF MATERIAL TO BE PUBLISHED OR PRESENTED
A multiplex quantitative analysis of secreted proteins in bronchoalveolar lavage samples from war veterans with chronic respiratory symptoms

2. FUNDING RECEIVED FOR THIS STUDY? ☒ YES ☐ NO **FUNDING SOURCE:** MOMRP

3. IS THIS MATERIAL CLASSIFIED? ☐ YES ☒ NO

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☐	**PUBLICATION/JOURNAL (List intended publication/journal)**
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☒	**POSTER (To be demonstrated at meeting/Name of Meeting, City, State, and Date of Meeting)** A multiplex quantitative analysis of secreted proteins in bronchoalveolar lavage samples from war veterans with chronic respiratory symptoms
☐	**PLATFORM PRESENTATION (At civilian institutions/Name of Meeting, State, Date of Meeting)**
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6. WHAT IS THE EXPECTED DATE YOUR PRESENTATION/PUBLICATION WILL BE SUBMITTED TO THE DEFENSE TECHNICAL INFORMATION CENTER (DTIC)? N/A

POINT OF CONTACT

7. WHO IS THE PRIMARY 59 MDW POINT OF CONTACT? (Last, First, MI.) (Include email)	DUTY PHONE/PAGER No.
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I CERTIFY ANY HUMAN OR ANIMAL RESEARCH RELATED STUDIES WERE APPROVED AND PERFORMED IN STRICT ACCORDANCE WITH 32 CFR 219, AFMAN 40-401_IP AND 59 MDWI 41-108. I HAVE READ THE FINAL VERSION OF THE ATTACHED MATERIAL AND CERTIFY THAT IT IS AN ACCURATE MANUSCRIPT FOR PUBLICATION AND/OR PRESENTATION.

AUTHOR'S PRINTED NAME/RANK/GRADE	AUTHOR'S SIGNATURE	DATE
Hui Xia, CTR, 59MDW/ST	XIA.HUI.1456595843 Digitally signed by XIA.HUI.1456595843 DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=CONTRACTOR, cn=XIA.HUI.1456595843 Date: 2016.11.16 11:55:24 -06'00'	Nov 16, 2016
APPROVING AUTHORITY'S PRINTED NAME, RANK, TITLE	**APPROVING AUTHORITY'S SIGNATURE**	**DATE**
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TO: Clinical Research Division (59 MDW/SGVU) (Contact 292-7141 for email instructions)	1. DATE RECEIVED	2. ASSIGNED PROCESSING REQUEST FILE NUMBER
		16395

3. DATE REVIEWED	4. DATE FORWARDED TO PA
1 Dec 2016	

5. AUTHOR CONTACTED FOR RECOMMENDED OR NECESSARY CHANGES

☒ NO ☐ YES If yes give date: ☐ N/A

6. COMMENTS

☒ APPROVED ☐ DISAPPROVED

The poster presentation is approved.

PRINTED NAME, RANK/GRADE, TITLE OF REVIEWER	DATE	SIGNATURE OF REVIEWER
Rocky Calcote, PhD, Clinical Research Administrator		CALCOTE.ROCKY.D.1178245844 Digitally signed by CALCOTE.ROCKY.D.1178245844 DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=CALCOTE.ROCKY.D.1178245844 Date: 2016.12.01 14:50:04 -06'00'

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	Dec 1, 2016	Dec 2, 2016

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PRINTED NAME, RANK/GRADE, TITLE OF REVIEWER	DATE	SIGNATURE OF REVIEWER
Kevin Iinuma, SSgt/E-5, 59 MDW Public Affairs	Dec 2, 2016	IINUMA.KEVIN.MITSUGU.1296227613 Digitally signed by IINUMA.KEVIN.MITSUGU.1296227613 DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=IINUMA.KEVIN.MITSUGU.1296227613 Date: 2016.12.02 07:31:54 -06'00'

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3. DATE WRITTEN NOTICE OF APPROVAL AND CLEARANCE MAILED TO AUTHOR:

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PRINTED NAME, RANK/GRADE, TITLE OF REVIEWER	SIGNATURE OF REVIEWER	DATE

A multiplex quantitative analysis of secreted proteins in bronchoalveolar lavage samples from war veterans with chronic respiratory symptoms

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ABSTRACT

A significant number of veterans who participated in Iraq and Afghanistan wars are inflicted with chronic lung problems. Many such active duty personnel and veterans have been the subjects of a study termed STAMPEDE – Study of Active Duty Military for Pulmonary Disease related to Environmental Deployment Exposure. However, so far no definitive causal relationships between the characteristic clinical and pathological lung conditions and extrinsic factors has been established. The current study aimed to analyze 37 cytokines and other secreted proteins in the bronchoalveolar lavage (BAL) samples from healthy as well as the patients. The lavage samples were centrifuged to pellet cells and other debris, and supernatants were saved for further analysis. The analysis employed the Luminex bead-based high-throughput approach to simultaneously analyze the level of each of the 37 secreted cytokines and other proteins. Total protein concentration in each sample was determined by a fluorescence method using the Qubit. The individual protein determinations in each sample were then normalized to the total protein in the same sample. The results show that for many of the proteins analyzed there was little or no difference in levels between the healthy and lung disease patients. However, for many others there were marked differences in levels. The proteins whose levels were about 1.5 to nearly 4 times higher in the lung disease samples than the healthy ones were sIL-6 receptor, BAFF, and IL-8. The proteins that showed higher levels in the control samples than the disease samples were APRIL (1.5 fold) and INF-beta (2 fold). Many others appeared to be at the same level in healthy and disease samples. These results suggest the possibility that secreted protein profile in these patients in comparison to the healthy individuals may help distinguish the lung conditions due to exposure in the war theater. However, more extensive studies would be needed.

INTRODUCTION

Military personnel who served in Iraq and Afghanistan are at an increased risk of developing respiratory symptoms, compared with non-deployed troops. According to a study involving more than 760,000 veterans, 6 percent of veterans have one or more chronic respiratory symptoms. Several studies have attributed the chronic respiratory disease to harsh environmental exposures, such as dust, burn pits and other chemical hazards. Many chronic respiratory disease, like asthma and chronic obstructive pulmonary disease, are inflammatory disease of the airways. Multiple cytokines play a key role in the inflammatory airway diseases. In chronic inflammation, the level of cytokines become unbalance. For example, the level of cytokines, such as THFalpha, IL-8, IFNgamma, is increased in asthma. In this study, we compared the level of inflammation cytokines in the respiratory disease from the patients who deployed to Iraq and Afghanistan to that seen in healthy subjects. The discovery of anti-inflammatory targets will be helpful for developing new therapeutic strategies in the chronic respiratory disease.

Table 1. Bio-Plex Pro Human Inflammation Panel 1, 37-Plex

APRIL/TNFSF13	IL-11	LIGHT/TNFSF14
BAFF/TNFSF13B	IL-12 (p40)	MMP-1
sCD30/TNFRSF8	IL-12 (p70)	MMP-2
sCD163	IL-19	MMP-3
Chitinase 3-like 1	IL-20	Osteocalcin
gp130/sIL-6R beta	IL-22	Osteopontin
IFN-alpha 2	IL-26	Pentraxin-3
IFN-beta	IL-27 (p28)	sTNF-R1
IFN-γ	IL-28A/IFN-lamda 2	sTNF-R2
IL-2	IL-29/IFN-lamda 1	TSLP
sIL-6R alpha	IL-32	TWEAK/TNFSF12
IL-8	IL-34	
IL-10	IL-35	

METHODS

SAMPLES

BAL (bronchoalveolar lavage) fluid samples from 38 healthy control subjects and 193 patient subjects were collected according to a standardized method.

PROTEIN QUANTIFICATION

Total protein in BAL fluid was measured using Qubit protein assay kits and the Qubit 3.0 fluorometer (ThermoFisher Scientific).

ASSAY

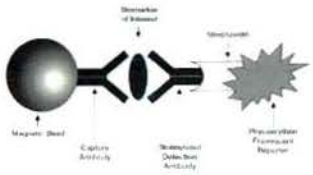
Cytokines in BAL fluid were quantified using the Bio-Plex Pro Human Inflammation Panel I, 37-Plex Assay kit (Bio-Rad Laboratories, Inc.) following the manufacture's recommendations. 50µl of each sample was assayed in duplicate on a 96 well plate, and then read on the Luminex MAGPIX Instrument (Luminex Corporation). Each 96 well plate included the following controls, which were assayed in duplicate: an 8 point standard curve, a negative blank control, and a positive control (provided by the manufacturer, Bio-Rad Laboratories, Inc.).

STATISTICAL ANALYSIS

For each of the 37 cytokines, the observed concentration of the cytokine (pg/mL) in each sample was divided by the total amount of protein (µg/mL) to calculate the final pg/µg value. The results of study were presented as means ± SEM. Group comparisons were done using an unpaired Student's t-test. Statistical signification defined as having a P value of less than 0.05.

Figure 1. A schematic depiction of the bead-based multiplex immunoassays for BAL fluid.

Figure 2. A depiction of the Bio-Plex sandwich immunoassay (Bio-Rad Laboratories, Inc.)

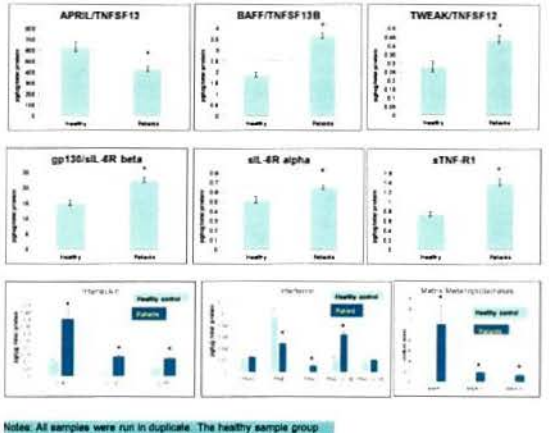


RESULTS

Table 2. Human inflammation biomarkers in BAL fluid between subjects with Chronic respiratory symptoms and healthy control.

Cytokines and Cellular component	Healthy BAL [n=38] Mean [range] pg/ml	Chronic Pulmonary Symptoms BAL [n=193] Mean [range] pg/ml
APRIL/TNFSF13	[illegible]	[illegible]
BAFF/TNFSF13B	[illegible]	[illegible]
sCD30/TNFRSF8	[illegible]	[illegible]
sCD163	[illegible]	[illegible]
Chitinase 3-like 1	[illegible]	[illegible]
gp130/sIL-6R beta	[illegible]	[illegible]
IFN-alpha 2	[illegible]	[illegible]
IFN-beta	[illegible]	[illegible]
IFN-γ	[illegible]	[illegible]
IL-2	[illegible]	[illegible]
sIL-6R alpha	[illegible]	[illegible]
IL-8	[illegible]	[illegible]
IL-10	[illegible]	[illegible]
IL-11	[illegible]	[illegible]
IL-12 (p40)	[illegible]	[illegible]
IL-12 (p70)	[illegible]	[illegible]
IL-19	[illegible]	[illegible]
IL-20	[illegible]	[illegible]
IL-22	[illegible]	[illegible]
IL-26	[illegible]	[illegible]
IL-27 (p28)	[illegible]	[illegible]
IL-28A/IFN-lamda 2	[illegible]	[illegible]
IL-29/IFN-lamda 1	[illegible]	[illegible]
IL-32	[illegible]	[illegible]
IL-34	[illegible]	[illegible]
IL-35	[illegible]	[illegible]
MMP-1	[illegible]	[illegible]
MMP-2	[illegible]	[illegible]
MMP-3	[illegible]	[illegible]
Osteocalcin	[illegible]	[illegible]
Osteopontin	[illegible]	[illegible]
Pentraxin-3	[illegible]	[illegible]
sTNF-R1	[illegible]	[illegible]
sTNF-R2	[illegible]	[illegible]
TSLP	[illegible]	[illegible]
TWEAK/TNFSF12	[illegible]	[illegible]

Figure 3. The comparison of Cytokine and Cellular component in BAL fluid between Healthy control and chronic respiratory symptoms.



Notes: All samples were run in duplicate. The healthy sample group includes 38 samples. The patient group includes 193 samples. The error bars represent the Standard Error of the Mean.
* P<0.01

DISCUSSION

1. Increased concentration of IFNγ in the patients, which is associated with T cell response from Th1 and Th2.
2. Increased concentration of Metalloproteinases (MMP1,2 and 3) in the patients, which can cause morphological changes in the lung.
3. Increased concentration of IL-8 and tumor necrosis factor superfamily (BAFF and TWEAK) in the patients.
4. Increased concentration of IL-19 in the patients, which was reported an increased level in the lungs of mice exposed to allergens .
5. Further assay for more cytokines will be finished.

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